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Article

Development of Interleukin-17-Producing γδ T Cells Is Restricted to a Functional Embryonic Wave

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SUMMARY

γδ T cells are an important innate source of interleukin-17 (IL-17). In contrast to T helper 17 (Th17) cell differentiation, which occurs in the periphery, IL-17-producing γδ T cells (γδT17 cells) are probably committed during thymic development. To study when γδT17 cells arise during ontogeny, we used *TcrdH2BeGFP* reporter mice to monitor T cell receptor (TCR) rearrangement and IL-17 production in the embryonic thymus. We observed that several populations such as innate lymphoid cells and early T cell precursors were able to produce IL-17 prior to (and thus independent of) TCR recombination. γδT17 cells were absent after transplantation of IL-17-sufficient bone marrow into mice lacking both *Il17a* and *Il17f*. Also, γδT17 cells were not generated after genetic restoration of defective *Rag1* function in adult mice. Together, these data suggested that these cells developed exclusively before birth and subsequently persisted in adult mice as self-renewing, long-lived cells.

INTRODUCTION

T cells expressing the γδ T cell receptor (TCR) are an important innate source of the proinflammatory cytokines interleukin-17 (IL-17A) and IL-17F (Bonneville et al., 2010; Cua and Tato, 2010; O'Brien et al., 2009). In mice, IL-17-producing γδ T cells (γδT17 cells) are abundant in secondary lymphoid organs, as well as in lung, liver, retina, and dermis (Cui et al., 2009; Kisielow et al., 2008; Lockhart et al., 2006; Murdoch and Lloyd, 2010; Simonian et al., 2009; Stark et al., 2005). In contrast to $CD4^+$ Th17 cells, γδT17 cells can immediately respond to a variety of stimuli including IL-23 and IL-1 and triggering of their TCR (Cua and Tato, 2010; Haas et al., 2009; Lalor et al., 2011; Mills, 2008). Accordingly, IL-17 production by γδ T cells has been shown to play important roles in the defense against mucocutaneous infections (Cho et al., 2010) and other microbial infections (Hamada et al., 2008; Lockhart et al., 2006; Ribot et al., 2009; Shibata et al., 2007). Furthermore, γδT17 cells are involved in the pathology of experimental autoimmune encephalomyelitis (Hirota et al., 2011; Petermann et al., 2010; Sutton et al., 2009), in anticancer immune responses (Hayday, 2009; Ma et al., 2011), and in the inflammatory processes associated with ischemic brain injury (Shichita et al., 2009).

In humans, it is currently emerging that the cytokines IL-17A and IL-17F are particularly important for control of mucocutaneous infections because their absence or mutations in their signaling pathway lead to chronic mucocutaneous candidiasis (Puel et al., 2011; van de Veerdonk et al., 2011). However, there is only sparse information about the contribution of γδ T cells to human IL-17 production (DeBarros et al., 2011; Deknuydt et al., 2009; Ness-Schwickerath et al., 2010; Peng et al., 2008). Of note, human γδT17 cells appear to be crucial for immune responses in young individuals (Caccamo et al., 2011; Moens et al., 2011).

Although it was shown that strong TCR engagement favors the development of γδ T cells precommitted to make interferon-γ (IFN-γ) (Jensen et al., 2008), it is unclear whether development of γδT17 cells is at all dependent on positive selection via TCR signaling. Current hypotheses suggest that development of γδT17 cells occurs either TCR independently (Jensen and Chien, 2009; Jensen et al., 2008) or requires a weak TCR signal (Turchinovich and Hayday, 2011). Recent literature suggests that γδT17 cells appear already in the embryonic thymus (Ribot et al., 2009) and their number progressively decreases in adult mice with age (Shibata et al., 2008). Although the embryonic ontogeny of γδ T cells is characterized by consecutive waves of γδ T cells with canonical combinations of Vγ and Vδ segments (Carding and Egan, 2002; Dunon et al., 1997), the ability to produce IL-17 does not appear to be restricted to cells with specific VγVδ chain pairs. Thus, γδT17 cells comprise semi-invariant $V\gamma6^+$ γδ T cells as well as more polyclonal $V\gamma1^+$ and $V\gamma4^+$ cells in lung, peritoneal cavity, and secondary lymphoid organs (Romani et al., 2008; Shibata et al., 2007; Simonian et al., 2009). Besides TCR engagement, other signals including interaction with CD4 and CD8 double-positive thymocytes (Powolny-Budnicka et al., 2011) or cytokines such as transforming growth factor-β (TGF-β) (Do et al., 2010) may mediate the transition of newly generated thymic $CD24^+CD44^{lo}$ γδ T cells to γδT17 cells displaying an activated $CD24^-CD44^{hi}$ effector phenotype.

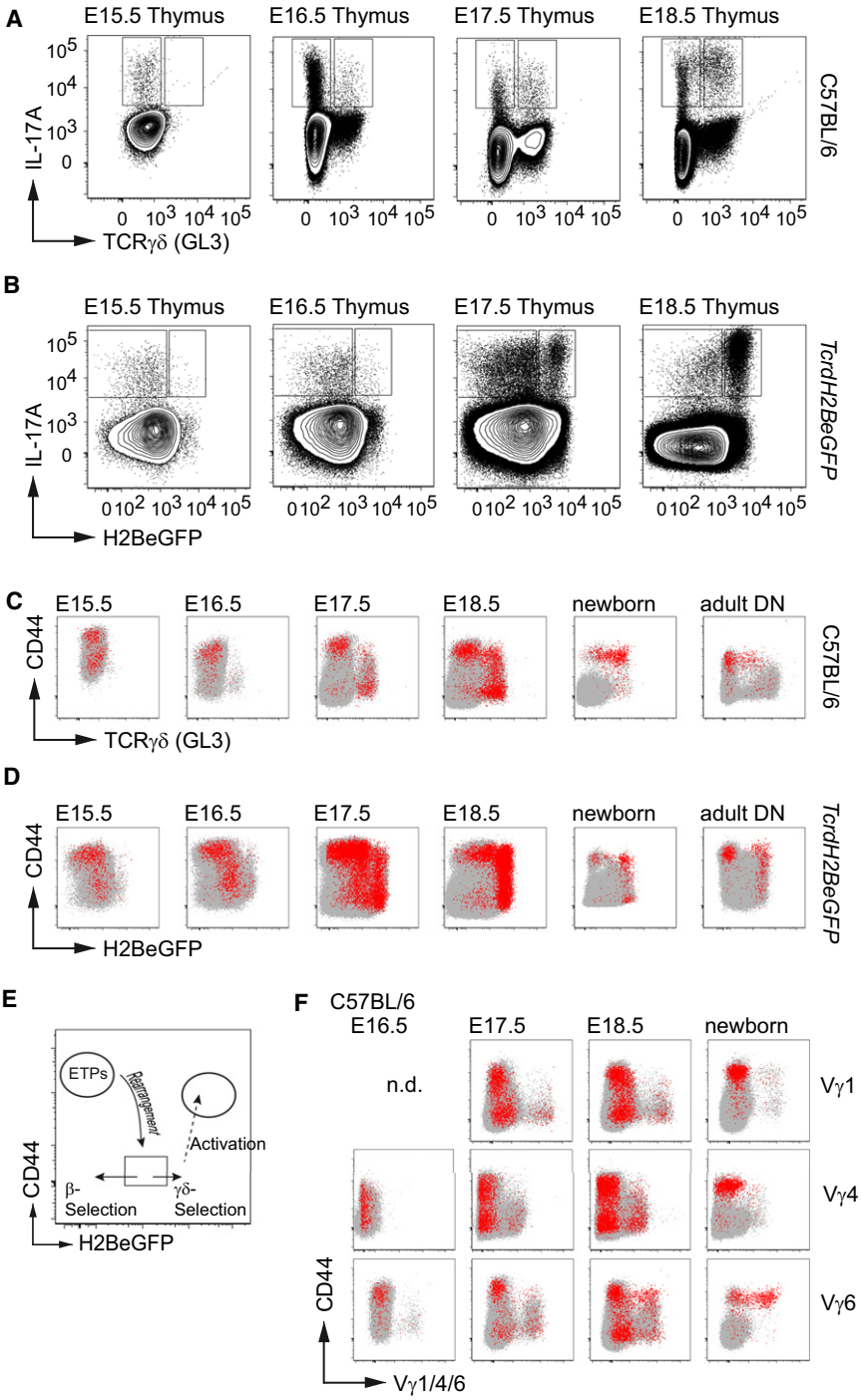

Figure 1. Development of IL-17-Producing γδ T Cells

(A and B) Dot plots show intracellular IL-17A staining of developing embryonic thymocytes from C57BL/6 (A) or *TcrdH2BeGFP* (B) mice at different days of gestation (E15.5–newborn) after PMA and ionomycin stimulation in the presence of brefeldin A.

(C and D) Overlays show gated IL-17$^+$ cells (red) in comparison to all cells (gray) from C57BL/6 (C) or *TcrdH2BeGFP* (D) mice on different days of gestation in a staining with CD44 versus the γδTCR by mAb staining (C, clone GL3) or *Tcrd*-reporter fluorescence (D).

(E) Schematic diagram depicts the different developmental stages of T cells from early thymic progenitors (ETPs, CD44^{hi}γδTCR$^-$) via TCR rearrangement and either β-selection or γδ T cell expression based on CD44 and *Tcrd*-reporter fluorescence.

(F) Overlays show gated IL-17$^+$ cells (red) in comparison to all cells (gray) on different days of gestation (E16.5 to newborn) in a staining with CD44 versus the respective Vγ chains (Vγ1, Vγ4, Vγ6).

Data are representative of one out of two independent experiments with 4–10 embryonic thymi per day. See also Figure S3.

To investigate the influence of IL-17 on the ontogeny of γδT17 cells, we produced mice deficient for both homologous cytokines IL-17A and IL-17F by targeting and deleting the entire *Il17af* locus (*Il17af*$^{-/-}$ mice). To monitor de novo development of γδ T cells in adult thymi, mice in which Rag1 deficiency could be reversed by conditional Cre-recombinase expression (Düber et al., 2009) were crossed with *TcrdH2BeGFP* reporter mice (Prinz et al., 2006). We observed that γδT17 cells were generated only within the embryonic thymus, suggesting that those which are found in adult mice are long-lived and self-renewing. Furthermore, our data may support the hypothesis that IL-17 produced by Th17 cells, γδT17 cells, and Thy1$^+$ innate lymphoid cells provides a negative feedback on the development and homeostasis of γδT17 cells. Together, our data suggest that the embryonic thymus provides a unique environment in which developing thymocytes can acquire an innate capacity to produce IL-17.

RESULTS

Development of IL-17-Producing γδ T Cells in the Embryonic Thymus

γδ T cells are frequent among the T cells that develop in the embryonic thymus (Havran and Allison, 1988). Also, γδ T cells are known to be potent producers of IL-17 (Roark et al., 2008). Here we used intracellular cytokine staining of thymocytes from embryonic day 15.5 to day 18.5 after ex vivo stimulation with phorbol myristate acetate (PMA) and ionomycin to find out when developing γδ T cells acquired the ability to produce IL-17. IL-17-producing thymocytes were already present at day E15.5 (Figure 1). Counterstaining with γδTCR mAb clone GL3 (Figure 1A) as well as the use of reporter fluorescence of the *Tcrd* locus in *TcrdH2BeGFP* mice (Figure 1B) revealed that γδ T cells with the capacity to produce IL-17 emerged around day E16.5 (Figures 1A and 1B). Furthermore, we observed that

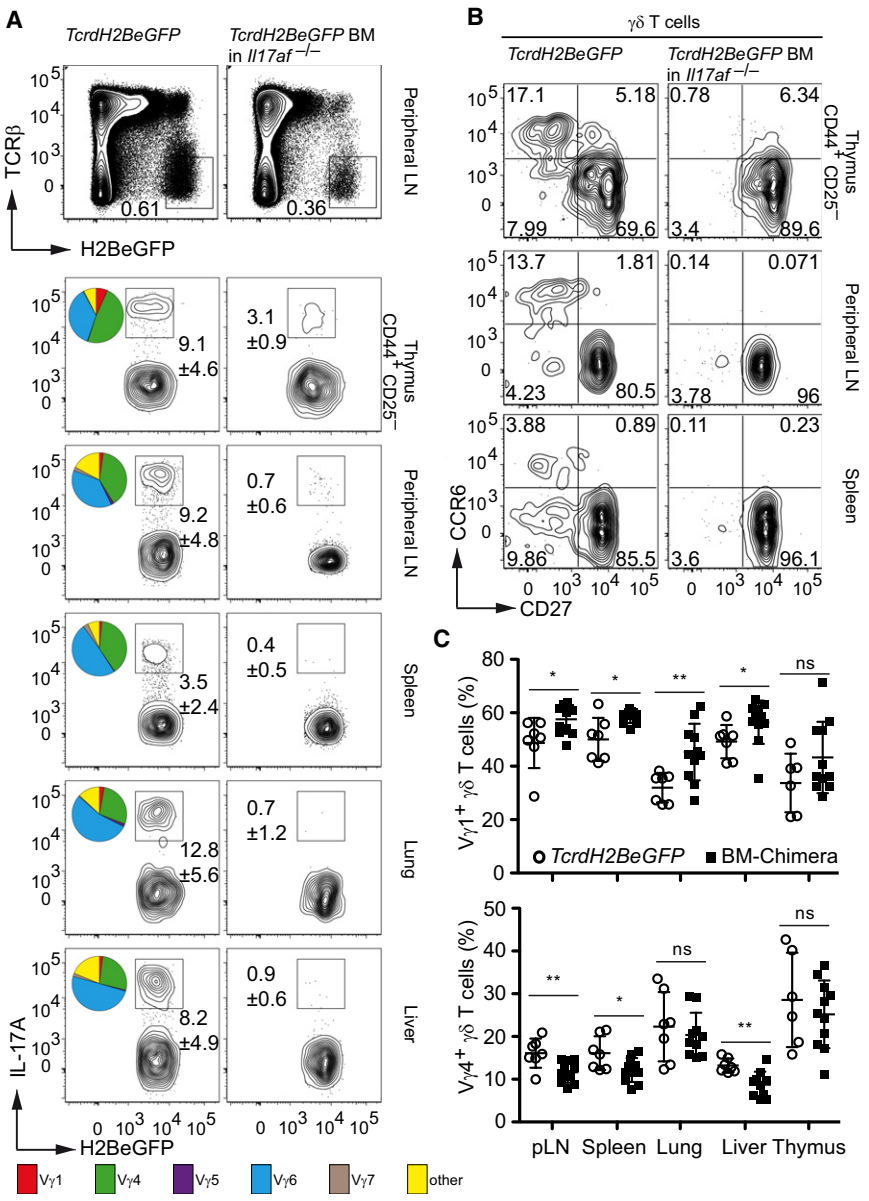

Figure 2. Reconstitution of IL-17-Producing αβ but Not γδ T Cells in *Il17af*$^{-/-}$ Hosts by Bone Marrow Transplantation with *TcrdH2BeGFP* γδ Reporter Mice

Lethally irradiated *Il17af*$^{-/-}$ mice were reconstituted with bone marrow from *TcrdH2BeGFP* mice. Organs were analyzed 8–14 weeks after bone marrow reconstitution.

(A) Representative intracellular IL-17A staining of γδ T cells in chimeras (right) compared to *TcrdH2BeGFP* mice (left) derived from indicated organs. Top panels exemplify the parent gates that identify H2BeGFP^{hi} γδ T cells. Thymic γδ T cells were additionally gated on CD44^{+}CD25^{-} cells. Pie charts in left panels show the relative contribution of the indicated Vγ chains to all IL-17^{+} gated γδ T cells.

(B) Flow cytometry analysis of gated γδ T cells in chimeras (right) compared to *TcrdH2BeGFP* mice (left) for expression of CCR6 and CD27.

(C) Frequency of either Vγ1 (top)- or Vγ4 (bottom)-expressing cells among all γδ T cells in untreated or chimeric mice.

Data are representative of four independent experiments with three to six mice per group. Either representative plots (B) or mean ± SD of four (A) or pooled data from two (C) experiments is shown. Statistically significant differences were tested with the Mann-Whitney test (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). See also Figure S2.

a fraction of fetal IL-17^{+} cells displayed a γδTCR^{-} phenotype (Figures 1C and 1D). Based on our previous findings that intermediate H2BeGFP expression indicates opening of the *Tcrd* locus for TCR rearrangement (Prinz et al., 2006), it is conceivable that developing T cell precursors with intermediate H2BeGFP expression were actually able to produce IL-17 prior to their final commitment to the γδ or αβ T cell lineage. A scheme how H2BeGFP versus CD44 expression allows monitoring of T cell development is depicted in Figure 1E. Notably, the presence of IL-17^{+} cells with intermediate amounts of the H2BeGFP-reporter fluorescence sharply declined at E18.5 and they were virtually absent in thymi from newborn and adult mice (Figure 1D). The TCR repertoire of developing IL-17^{+} γδ T cells was biased toward TCR containing Vγ4 and Vγ6 chains but also included Vγ1^{+} TCR (Figure 1F). Together, these observations suggest that the embryonic thymus between E15.5 and E18.5 provides a unique window for γδT17 cell development, possibly independent of TCR rearrangement.

Reconstitution of Peripheral IL-17-Producing αβ but Not γδ T Cells in *Il17af*$^{-/-}$ Hosts by Bone Marrow Chimerism

Next, we tested whether development of γδT17 cells was confined to the embryonic thymus. To this end, we transplanted bone marrow from IL-17-sufficient donors into IL-17-deficient adult recipient mice. To avoid redundancy of the highly homologous cytokines IL-17A and IL-17F, which are often coregulated and bind as homo- and heterodimers to common receptors (Iwakura et al., 2011), we generated recipient mice deficient for both cytokines. This was achieved by deleting a stretch of 46 kb in chromosome 1 comprising exons 2 and 3 of the syntenic genes *Il17a* and *Il17f* in C57BL/6-derived embryonic stem cells (*Il17af*$^{-/-}$ mice, see Figure S1 online available). 8–14 weeks after reconstitution of lethally irradiated *Il17af*$^{-/-}$ mice with bone marrow from *TcrdH2BeGFP* mice, γδ T cells were readily detected in thymus and secondary lymphoid organs, as well as in lung and liver of recipients (Figure 2A). However, γδT17 cells were largely missing in these chimeras (Figure 2A). Likewise, γδT17 cells were virtually absent in chimeric *Il17af*$^{-/-}$ mice that received bone marrow from C57BL/6 wild-type (WT) mice, whereas absolute numbers and frequencies of IL-17-producing αβTCR^{+} lymphocytes were similar to WT controls (Figures

S2A–S2C). A notable exception was the thymus, where we always detected a sizable proportion of γδ T cells capable of IL-17 production (Figure 2A). However, these thymic IL-17$^+$ cells in chimeras did not adopt the typical CCR6$^+$CD27$^-$ phenotype of genuine γδT17 cells (Figure 2B; Haas et al., 2009). Consistently, donor-derived CCR6$^+$CD27$^-$ γδ T cells were also absent in the periphery of bone marrow chimeras (Figure 2B). As predicted from analysis of the TCR repertoire of γδT17 cells in embryonic thymi (Figure 1F), the majority of γδT17 cells in adult mice displayed either Vγ4 or Vγ6 TCR chains in all organs analyzed but this was not exclusive; a few γδT17 cells expressed other Vγ chains (Figure 2A, left column insets). Rearrangement of the canonical Vγ6$^+$Vδ1$^+$ TCR occurs mainly in the embryonic thymus (Carding and Egan, 2002; Dunon et al., 1997) and as will be shown later, Vγ6$^+$ cells do not efficiently develop in adult thymi. Thus it was not surprising that the Vγ6$^+$ fraction of γδT17 cells were absent in bone marrow chimeras. In contrast, both Vγ1$^+$ and Vγ4$^+$ γδ T cells were efficiently regenerated in the chimeras (Figure 2C) but failed to show the cytokine profile or CCR6$^+$CD27$^-$ phenotype of γδT17 cells. However, there was a shift from Vγ4 to increased Vγ1 chain usage among the regenerated H2BeGFP$^+$ donor γδ T cells compared to untreated control mice (Figure 2C).

To narrow down the temporal window that allows for development of effector γδT17 cells, we investigated whether a neonatal thymic environment in newborn mice still supported the development of γδT17 cells. Therefore, neonatal *Il17af*$^{-/-}$ mice received intrahepatic injections of lineage-negative bone marrow cells from *TcrdH2BeGFP* mice. In line with our results from bone marrow chimeras above, we observed that some donor-derived H2BeGFP$^+$ γδ T cells were able to produce IL-17 in the thymus but not in the periphery (Figure S3A). However, overall reconstitution of CCR6$^+$CD27$^-$ effector γδT17 cells was not possible by neonatal transfer of T cell precursors (Figure S3B).

Next, we addressed whether embryonic T cell precursors derived from day E13.5 fetal liver would be sufficient to sustain the development of γδT17 cells in adult *Il17af*$^{-/-}$ mice (Figure S3C). Again, we found no mature CCR6$^+$CD27$^-$ effector γδT17 cells although some donor-derived γδ T cells were able to produce IL-17 in the thymus (Figure S3C). Together, these data suggest that functional CCR6$^+$CD27$^-$ effector γδT17 cells can not be regenerated after bone marrow transplantation in adult mice.

Development of γδT17 Cells Requires an Embryonic Thymus

To exclude potential irradiation effects and other confounding factors arising during hematopoietic reconstitution, we next employed an alternative system to test whether γδ T cells developing in adult mice could acquire the capacity to produce IL-17. We crossed *TcrdH2BeGFP*-reporter mice to recombination-activating gene 1 (*Rag1*)-targeted mice (Düber et al., 2009), in which T and B cell development can be induced by tamoxifen gavage (Indu-*Rag1* × *Rosa26creERT2*, here termed Indu-*Rag1*) (Figure 3A). Induction of *Rag1* expression in adult Indu-*Rag1* × *TcrdH2BeGFP* mice permitted T cell development (Figures S4A and S4B) and led to the rapid emergence of H2BeGFP$^+$ γδ T cells in secondary lymphoid organs (Figure 3B). Within 2 weeks of *Rag1* induction, a large proportion of peripheral lymph node (LN) cells were γδ T cells (ranging from 2% to 10% of all lymphocytes) (Figure 3B). This high frequency might be due to faster development of γδ T cells compared to αβ T cells that need to pass through further thymic selection stages. In line with our observations in bone marrow chimeras above, induction of *Rag1* expression in adult mice led to the generation of both Vγ1$^+$ and Vγ4$^+$ γδ T cells but Vγ6$^+$ T cells were largely absent (Figures 3C and S4C). As previously described for bone marrow chimeras, Vγ7$^+$ intestinal intraepithelial lymphocytes could be fully regenerated (Figure 3D; Chennupati et al., 2010). In contrast, resident dendritic epidermal γδ T cells (DETCs), regarded as prototype "embryonic wave" of specialized γδ T cells, could not be generated after *Rag1* induction (Figure 3E). Notably, we found that *Rag1* induction in adult mice could not induce de novo development of effector γδT17 cells (Figure 3F). Accordingly, CCR6$^+$CD27$^-$ γδ T cells were absent after *Rag1* induction (Figure S4D). Consistent with our observations in bone marrow chimeras, Indu-*Rag1* × *TcrdH2BeGFP* mice showed a shift from Vγ4$^+$ to increased Vγ1$^+$ cells (Figures S4E and S4F). Taken together with the bone marrow chimeras, we demonstrate that γδT17 cells are not produced in adult thymi in two complementary experimental systems.

Finally, we evaluated the development of γδT17 cells in an embryonic and adult thymus side by side within the same animal. To this end, we performed transplantations of embryonic day E15.5 thymic lobes extracted from congenic CD45.1$^+$ WT mice under the kidney capsule of adult Indu-*Rag1* × *TcrdH2BeGFP* mice and simultaneously induced *Rag1* expression in host T cell precursors by tamoxifen gavage (Figures 4 and S5A). After 2 weeks, CD45.2$^+$ thymocytes of host origin including CD4 and CD8 double and single positives could be detected in the grafted thymic lobe (Figure S5B). After 5 weeks, already >97% of thymocytes in grafted thymus were derived from CD45.2$^+$ host precursors (Figure S5C). Of note, comparison of thymus-graft-derived CD45.1$^+$H2BeGFP$^-$ and host-derived CD45.2$^+$H2BeGFP$^+$ γδ T cells revealed that approximately 30%–50% of donor γδ T cells but none of host origin displayed a CCR6$^+$CD27$^-$CD44^{hi} phenotype (Figure 4A) and were committed to produce IL-17 (Figures 4B and 4C). We noted a CCR6$^+$CD44^{lo} γδ T cell population derived from the adult progenitors, but these failed to produce IL-17 upon stimulation. These cells might represent an intermediate stage that failed to complete γδT17 cell differentiation. The few CD45.2$^+$ effector γδT17 cells detected in the periphery of grafted mice were not significantly above background numbers detected in sham operated mice (Figure 4C). Five weeks after transplantation, CCR6$^+$CD27$^-$CD44^{hi} cells present in the adult mouse had exclusively developed from embryonic donor thymocytes being present during transplantation and those were therefore long-lived. These data suggest that entrance of adult T cell precursors into the transplanted embryonic thymi was either too late to undergo an embryonic differentiation program or that these were intrinsically different compared to those few precursors already present at E15.5 in the transplanted donor thymus, or both.

IL-17 Controls the Homeostasis and Generation of CCR6$^+$CD27$^-$ Effector γδ T Cells

We next investigated the mechanisms that control development and homeostasis of CCR6$^+$CD27$^-$ γδT17 cells. Interestingly,

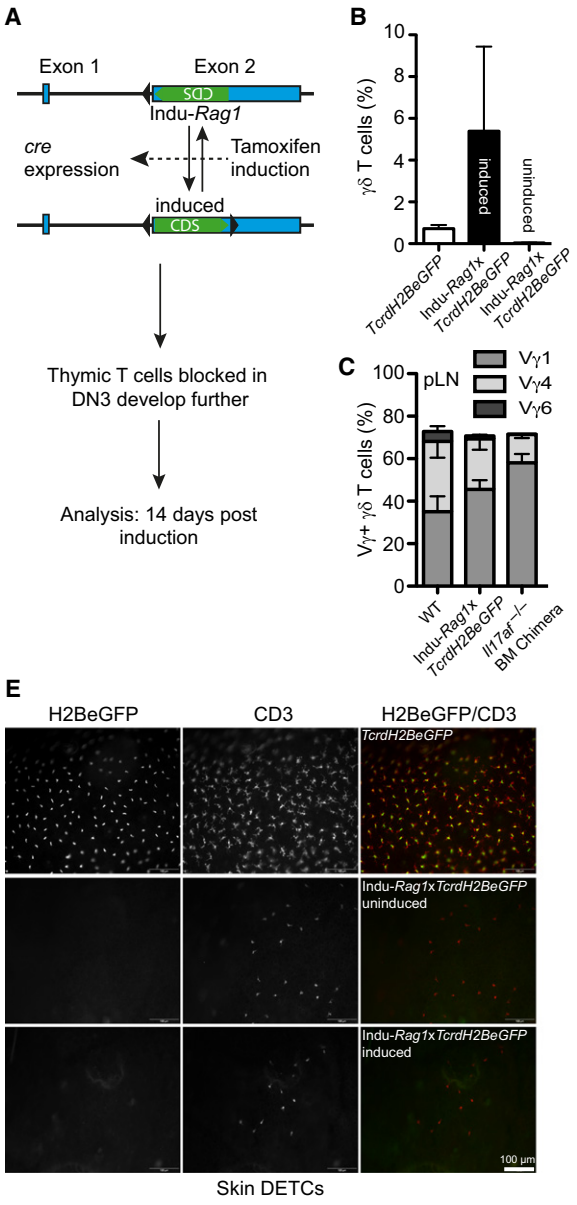

Figure 3. Induction of T Cell Development in Adult Indu-*Rag1* × *TcrdH2BeGFP* Mice Does Not Give Rise to γδT17 Cells, but to IFN-γ-Producing γδ T Cells

(A) Schematic description of *cre* induction for the recombination of exon 2 of the *Rag1* gene. Tamoxifen induction of *cre* expression rescues *Rag1* gene expression in T cells whose development was blocked in the double-negative 3 stage (DN3) in the thymus.

(B–E) Indu-*Rag1* × *TcrdH2BeGFP* mice were induced with tamoxifen for 2 weeks at the age of 7–10 weeks when analyzed.

(B) Frequencies of γδ T cells among lymphocytes in peripheral LNs in *TcrdH2BeGFP* mice, induced or uninduced Indu-*Rag1* × *TcrdH2BeGFP* mice.

(C) Frequencies of different Vγ chains among γδ T cells in either untreated *TcrdH2BeGFP*, induced Indu-*Rag1* × *TcrdH2BeGFP* mice, or bone marrow chimeras.

(D) Intestinal intraepithelial lymphocytes (iIELs) were isolated from the small intestines from either *TcrdH2BeGFP*, induced, or uninduced Indu-*Rag1* × *TcrdH2BeGFP* mice and stained for Vγ7Vδ6.3. Contour plots show gated γδ T cells detected by their TCRβ$^-$H2BeGFP^{hi} expression.

(E) Histological analysis of epidermal sheets from ears of either *TcrdH2BeGFP*, induced, or uninduced Indu-*Rag1* × *TcrdH2BeGFP* mice stained for CD3. H2BeGFP reporter fluorescence expression is shown in green.

(F) Intracellular staining for IL-17A$^+$ in gated γδ T cells from Indu-*Rag1* × *TcrdH2BeGFP* mice (lower) compared to *TcrdH2BeGFP* mice (top).

Data are representative from two (C, E), three (D, F), or four (B) independent experiments with 2–3 (D–F) or 3–5 (B) mice per group. (B), (C), and (D) show mean ± SD. See also Figure S4.

a series of observations and experiments collectively pointed to a critical role of IL-17 itself. An important clue came from the analysis of *Il17af*$^{-/-}$ mice. These show higher frequencies and absolute numbers of γδ T cells with a CCR6$^+$CD27$^-$ effector phenotype in thymus and peripheral organs when compared to IL-17-sufficient littermates (Figures 5A and 5B). Elevated frequencies of γδ T cells among all lymphocytes in the absence of IL-17 were due to a selective increase of CCR6$^+$CD27$^-$ γδ T cells whereas absolute numbers of CCR6$^-$CD27$^+$ γδ T cells were not altered (Figures 5B and 5C). Proliferation analysis revealed a moderately higher turnover of CCR6$^+$CD27$^-$ γδ T cells in *Il17af*$^{-/-}$ compared to littermates. No difference was observed for CCR6$^-$CD27$^+$ γδ T cells, of which only <5% incorporated bromodeoxyuridine (BrdU) during 4 days in the periphery (Figure 5D). Only in the thymus, CCR6$^-$CD27$^+$ γδ T cells showed an increased BrdU uptake, probably because this pool (in contrast to CCR6$^+$ γδ T cells) was constantly replenished by thymic de novo generation (Figure 5D). Thus, a high turnover of CCR6$^+$CD27$^-$ γδ T cells might reflect their self-renewing capacity, particularly in the absence of IL-17. With the obvious exception of lacking IL-17 production, the CCR6$^+$CD27$^-$ γδ T cells in *Il17af*$^{-/-}$ mice were a phenocopy of WT CCR6$^+$CD27$^-$ γδ T cells. Both did not produce IFN-γ and were NK1.1$^-$ but Rorγt$^+$ (Figures S6A–S6C). Deficiency of IL-17 was not compensated by increased expression of IL-22 in knockout cells (Figure S6D).

To further assess the role of IL-17 in γδT17 cell development, we next reconstituted lethally irradiated Thy1.1$^+$ *Il17af*$^{-/-}$ mice with lineage-negative bone marrow derived from Thy1.2$^+$ *Il17af*$^{-/-}$ mice. We found that in the complete absence of IL-17, donor-derived CCR6$^+$CD27$^-$ effector γδ T cells could indeed be regenerated in adult mice making up to 2.9% and 4.0% in peripheral LN and spleen, respectively (Figure 6A, right column). These frequencies are still lower than in untreated WT mice but clearly higher than in chimeras reconstituted with IL-17-sufficient bone marrow (Figure 6A, left column). Furthermore, we noted an increased proportion of CCR6$^+$CD27$^-$ γδ

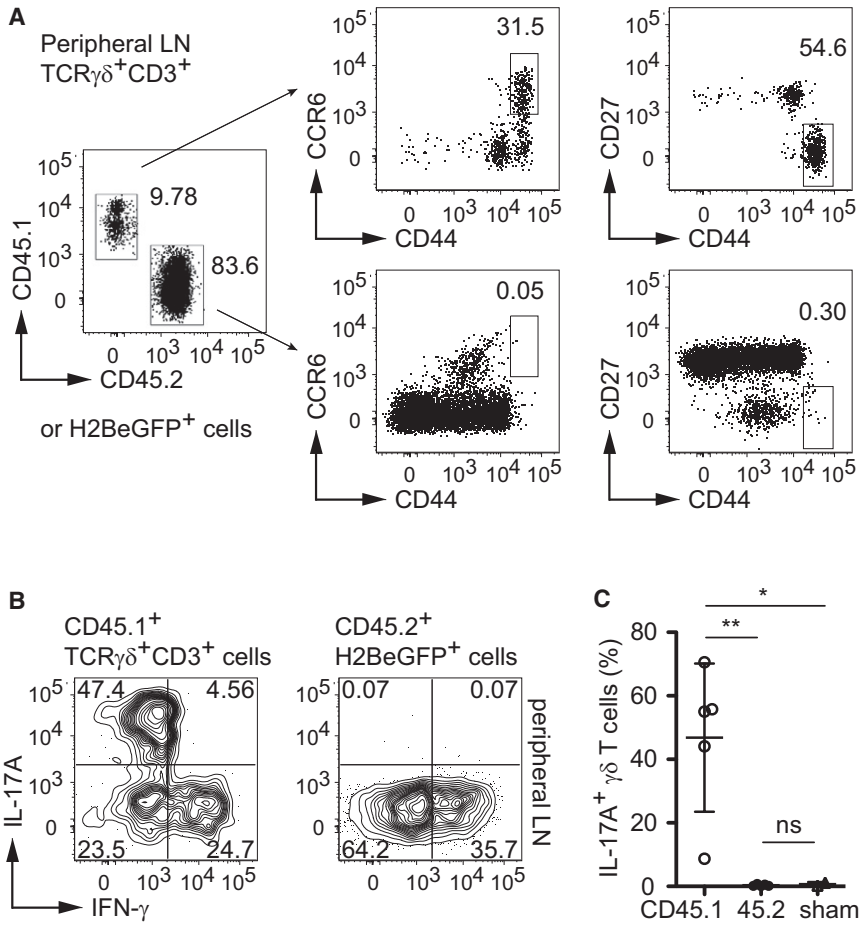

Figure 4. The Embryonic Thymus Is Necessary for the Development of γδT17 Cells

Embryonic thymi from E15.5 C57BL/6 (CD45.1) mice were transplanted under the renal capsule of tamoxifen-induced Indu-*Rag1* × *TcrdH2BeGFP* mice (age: 10 weeks) and analyzed 5 weeks later.

(A) Dot plots show flow cytometry data of surface CCR6, CD27, and CD44 staining of CD45.1$^+$ or CD45.2$^+$ γδ T cells identified either by their γδTCR expression (CD45.1$^+$GL3$^+$CD3$^+$) or by their intrinsic *H2BeGFP* reporter gene expression (CD45.2$^+$ cells).

(B) Contour plots show intracellular IL-17A and IFN-γ staining of γδ T cells from peripheral lymph nodes gated on either CD45.1$^+$ γδ T cells or CD45.2$^+$ γδ T cells.

(C) Scatter plots show percentages of IL-17A$^+$ cells among embryonic thymus-derived γδ T cells (CD45.1) and host-derived γδ T cells (CD45.2) compared to sham operated mice.

Data are pooled from one of three independent experiments with 1–3 mice per group and the mean ± SD is shown (C). Statistically significant differences were tested with a one-way ANOVA with Tukey post-test (*$p < 0.05$; **$p < 0.01$). See also Figure S5.

T cells among the residual host-derived Thy1.1$^+$ cells that survived lethal irradiation (Figure 6B) as compared to untreated mice (compare Figures 2B and 5C). This suggests that CCR6$^+$CD27$^-$ γδ T cells in peripheral lymphoid organs are relatively radioresistant, similar to and presumably exchanging with a recently identified population of radioresistant IL-17-producing dermal γδ T cells (Sumaria et al., 2011). In conclusion, the presence of γδ T cells with a characteristic CCR6$^+$CD27$^-$ phenotype of IL-17-producing γδ T cells in *Il17af*$^{-/-}$ mice shows that the cytokines IL-17A and IL-17F are not required for their development. To the contrary, an overabundance of these cells in the knockout as well as some albeit inefficient adult development of CCR6$^+$CD27$^-$ γδ T cells in the absence of IL-17 suggest that IL-17 itself may control the development and homeostasis of γδT17 cells.

Restriction of Thymic γδT17 Cell Development by IL-17-Producing αβ T Cells and Innate Lymphoid Cells

Next, we explored the sources of IL-17, which may be restricting de novo development of γδT17 cells in adult thymi. Therefore, we reconstituted groups of lethally irradiated *Il17af*$^{-/-}$ mice with 1:1 mixtures of either *Il17af*$^{-/-}$ and WT (Figure 6C, middle) or *Il17af*$^{-/-}$ and *Tcra*$^{-/-}$ (Figure 6C, right) bone marrow. The latter chimeras lacked IL-17-proficient αβ T cells because normal αβ T cell development was possible only from *Il17af*$^{-/-}$ precursors. Notably, these chimeras allowed adult generation of γδT17 cells to some extent (Figure 6C, right). This suggests that the presence of IL-17-producing αβ T cells may directly or indirectly inhibit homeostasis and development of γδT17 cells. A possible mechanism could involve a negative feedback through IL-17 as it was recently proposed to account for the expansion of IL-17A-producing T cells in IL-17RA-deficient mice (Smith et al., 2008). Indeed, we were able to detect expression of IL-17RC, the specific receptor subunit for IL-17A and IL-17F, in CCR6$^+$CD27$^-$ but not in CCR6$^-$CD27$^+$ γδ T cells (Figure 6D). These data further support the hypothesis that inhibition of γδT17 cell development may actually depend on IL-17. However, this raised the question of what would be the supply of IL-17 in uninduced Indu-*Rag1* mice where γδT17 cells were also not properly produced after tamoxifen-induced *Rag1* expression in adult mice. Looking for alternative sources of IL-17 that inhibited thymic development of γδT17 cells, we identified a previously unrecognized population of IL-17-producing Thy1$^+$TCRβ$^-$ thymocytes in Rag1-deficient and uninduced Indu-*Rag1* mice (Figures 7A–7C). Further characterization of these IL-17-producing cells revealed a CD44^{hi}CD24$^-$ phenotype, reminiscent of "cluster B" effector γδ T cells (Haas et al., 2009; Prinz et al., 2006), and they expressed CCR6 but not NK1.1 or CD117 (Figure 7D). Moreover, these cells showed striking similarities with intestinal innate lymphoid cells present in inflamed colons of Rag1-deficient mice (Buonocore et al., 2010) as they stained positive for Sca-1 and negative for CD4 (Figure 7E). IL-17-producing cells in the thymus of Rag1-deficient *TcrdH2BeGFP* reporter mice expressed the IL-7Rα chain CD127 and displayed an intermediate expression of

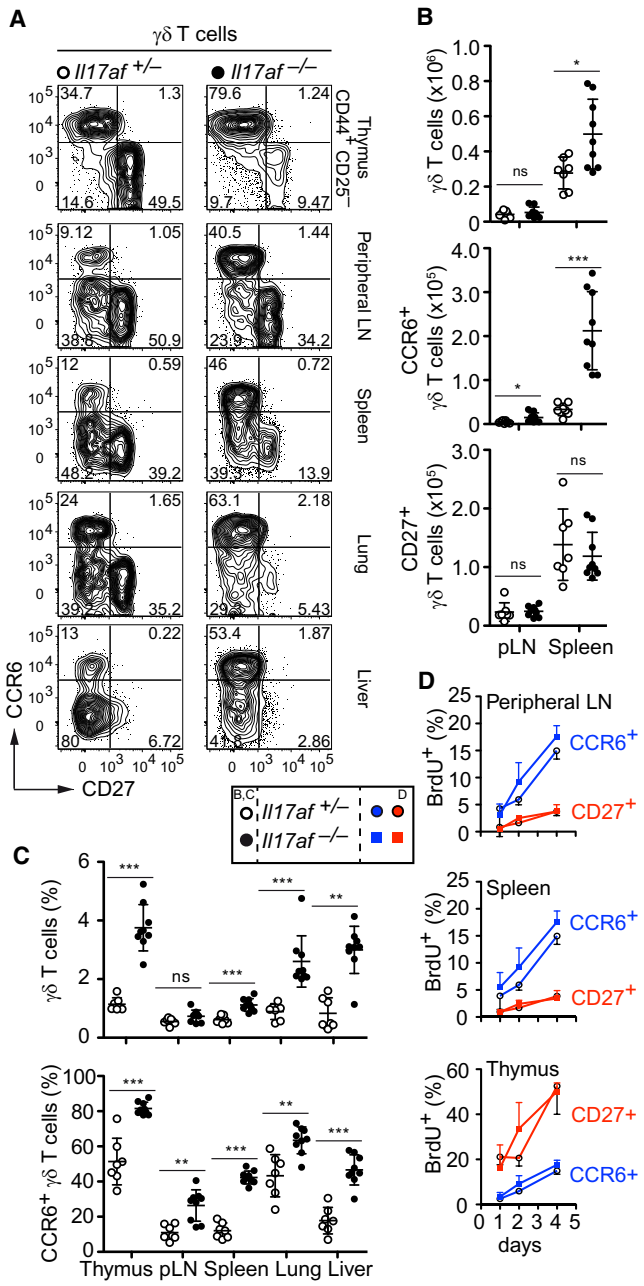

Figure 5. Deficiency of IL-17A and IL-17F Boosts the Amount of CCR6$^+$CD27$^-$ Effector γδ T Cells

(A) Surface CCR6 and CD27 staining of γδ T cells derived from the indicated organs of *Il17af*$^{-/-}$ mice (right) or heterozygous littermate *Il17af*$^{+/-}$ control animals (left).

(B and C) Absolute cell numbers or percentages of gated γδ T cells, CCR6$^+$ CD27$^-$ (B, C), or CCR6$^-$CD27$^+$ γδ T cells (B) from *Il17af*$^{-/-}$ mice (closed circles) are compared to heterozygous littermate *Il17af*$^{+/-}$ controls (open circles). Data are representative from one (A) or pooled from two (B, C) independent experiments of >5 independent experiments with 4–6 mice per group and mean ± SD is shown (B, C).

(D) Percentages of BrdU$^+$ γδ T cells in thymus, spleen, and peripheral lymph nodes (pLNs) among CCR6$^+$CD27$^-$ (blue) or CD27$^+$CCR6$^-$ (red) cells after continuous administration of BrdU for 4 days from *Il17af*$^{-/-}$ mice (colored squares) are compared to heterozygous littermate *Il17af*$^{+/-}$ controls (colored circles).

Data represent three to four mice per group and data point. Mean ± SD is shown (D). Mice were 8–12 weeks old. Statistically significant differences were tested with the Mann-Whitney test (*p < 0.05; **p < 0.01; ***p < 0.001). See also Figure S6.

H2BeGFP (Figure 7F). This indicated that these innate IL-17-producing cells in the thymus of Rag1-deficient mice had opened their *Tcrd* locus (Prinz et al., 2006) and were in a developmental stage attempting to undergo TCR rearrangement. In summary, this study shows that only the fetal thymus provides a window of opportunity for the development of γδT17 cells. These cells appear to acquire their capacity to produce IL-17 largely independent of TCR rearrangements.

DISCUSSION

In this work, we show that γδ T cells acquire the capacity to produce IL-17 in the embryonic thymus. After their development, γδT17 cells disseminate in peripheral organs including LN, spleen, liver, and lung, and some stay in the thymus (or recirculate). They are likely to comprise a recently identified population of IL-17-producing dermal γδ T cells (Gray et al., 2011; Kisielow et al., 2008; Sumaria et al., 2011). Despite their uniform CCR6^{+-} CD44^{hi}CD27$^-$ phenotype, populations of natural effector γδT17 cells are restricted neither to a particular canonical TCR chain usage nor to a specific anatomical location. Although γδT17 cells are mainly Vγ4$^+$ and Vγ6$^+$, other TCR rearrangements are also observed. This puts them in contrast to established "waves" of γδ T cell development, which are defined by invariant TCR rearrangements and by specific organ tropism (Carding and Egan, 2002; Dunon et al., 1997). Those "classical γδ T cell waves" include Vγ5$^+$Vδ1$^+$ DETCs (Asarnow et al., 1988; Havran and Allison, 1988), Vγ6$^+$Vδ1$^+$ intraepithelial cells of tongue and female reproductive tract (Itohara et al., 1990), Vγ7$^+$ intraepithelial cells of the small intestine (Asarnow et al., 1989; Itohara et al., 1990; Takagaki et al., 1989), and Vγ1$^+$Vδ6$^+$ cells in the liver (Gerber et al., 1999). On the contrary, generation of γδT17 cells appears to be best described as a polyclonal "functional embryonic wave," defined rather by a temporal window of opportunity than by specific Vγ chain usage. Whereas most Vγ6$^+$ cells display a γδT17 phenotype, Vγ4$^+$ cells are more heterogenous and the IL-17A$^-$IFN-γ$^+$ fraction of Vγ4$^+$ cells can be efficiently generated in adult mice. Thus, global gene expression analyses of γδ T cell subsets as recently published by the ImmGen consortium (Narayan et al., 2012) may benefit from further refinement. In addition to γδ T cell classification by Vγ usage, future approaches may yield a sharper delineation of functional subsets if these were sorted according to expression of markers such as CCR6 and CD27.

Several experimental strategies, including comparison of simultaneous de novo development of T cells in an adult and an ectopic embryonic thymus within the same animal, suggested that physiological development of CCR6$^+$CD27$^-$ γδT17 cells is terminated before birth and that these cells subsequently persist as a long-lived, self-renewing population. Such long-term persistence even in the thymus reconciles our data with a previous study by Shibata et al. (2008) on the ontogeny of γδT17 cells, which reported a peak of γδT17 cells in the perinatal period

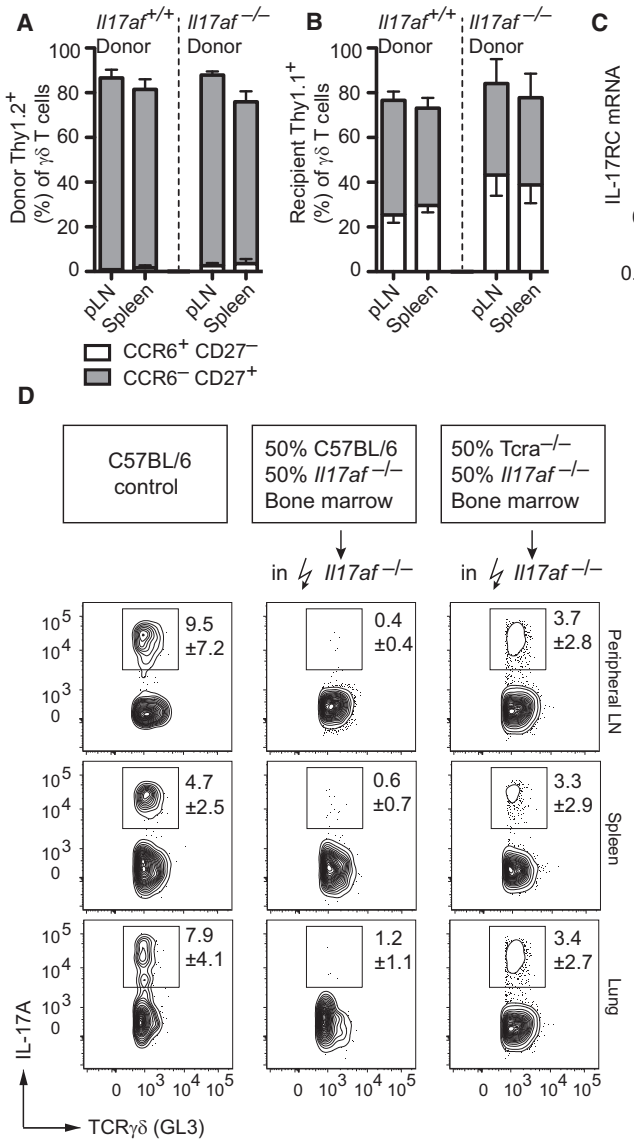

Figure 6. IL-17 Induces a Negative Feedback on IL-17-Producing γδ T Cells

(A and B) Lethally irradiated *Il17af*$^{-/-}$ mice (Thy1.1^{+}, recipients) were reconstituted with Thy1.2^{+}, donor bone marrow from *Il17af*$^{+/+}$ (left) or from *Il17af*$^{-/-}$ (right). After 8–10 weeks, donor Thy1.2^{+} (A) or residual recipient Thy1.1^{+} (B) cells were separately analyzed for frequencies of CCR6^{+}CD27^{-} or CCR6^{-}CD27^{+} γδ T cells.

(C) Real-time qPCR with primers for *Il17rc* on ex vivo sorted CCR6^{+}CD44^{hi} and CD27^{+}CCR6^{-} γδ T cells from mixed spleen and peripheral lymph nodes of either C57BL/6 or *Il17af*$^{-/-}$. Each dot represents one independent experiment with 15–20 mice per sort.

(D) Lethally irradiated *Il17af*$^{-/-}$ mice were reconstituted with either a 1:1 mix of WT and *Il17af*$^{-/-}$ bone marrow (middle) or a 1:1 mix of *Tcra*$^{-/-}$ and *Il17af*$^{-/-}$ bone marrow (right) and were compared to control animals (left). Intracellular IL-17A staining of gated γδ T cells in peripheral lymph nodes (LNs), spleen, lung, and liver. During bone marrow reconstitution, mice were 8–12 weeks old.

Error bars indicate SD (A–C). Data are representative from one (A, B) or three (C, D) independent experiments with four to five mice per group.

but also found these cells in the thymus of 2-month-old mice. In fact, extensive thymic persistence was also found for classical CD1d-restricted NKT cells (Benlagha et al., 2005) and for innate-like Thy-1^{lo}NK1.1^{+} Vγ1^{+}Vδ6^{+} cells (Grigoriadou et al., 2003). Thus, it appears to be a typical feature of innate lymphocytes. Furthermore, our study revealed a higher turnover of CCR6^{+}CD27^{-} γδT17 cells compared to CCR6^{-}CD27^{+} γδ T cells. At first sight, this is at odds with a former study (Ribot et al., 2009). Whereas that study investigated the expansion potential of CD27^{-} and CD27^{+} γδ T cells after ex vivo stimulation, our data reflected steady-state proliferation in vivo.

In addition to longevity and self-renewal, analysis of bone marrow chimeras revealed that CCR6^{+}CD27^{-} γδT17 cells were relatively radioresistant compared to CD27^{+} γδ T cells. This may explain the recently discovered radioresistance of dermal γδ T cells (Gray et al., 2011; Sumaria et al., 2011), among which γδT17 cells are very frequent. Persistence, longevity, and self-renewal together shape a stem cell-like phenotype for γδT17 cells, which is an interesting parallel to a recent study that portrayed a stem cell-like molecular signature in Th17-polarized αβ T cells (Muranski et al., 2011).

The present work establishes that IL-17-producing γδ T cells cannot be efficiently generated in adult mice. However, there are notable exceptions to this rule. It was recently proposed that intravenous injection of neonatal thymocyte suspensions could restore γδT17 cells in adult recipient mice (Gray et al., 2011) and this potential was attributed to neonatal T cell precursors. An alternative interpretation of those results might be that their adoptively transferred suspension already contained the matured cell population, i.e., long-lived and self-renewing γδT17 cells and not their precursors. In our study, a sizable proportion of thymic but not peripheral γδ T cells were apparently able to produce IL-17 in bone marrow chimeras or after *Rag1* induction in adult Indu-*Rag1* × *TcrdH2BeGFP* mice but did not acquire the CCR6^{+}CD27^{-} surface phenotype of mature IL-17 producers. It is currently unclear whether these cells failed to undergo complete differentiation and thus could not leave the thymus or whether they adopted a different phenotype before doing so. A hint may come from a recent study showing that thymic IL-17-producing γδ T cells developed in the absence of RORγt but failed to populate the periphery (Shibata et al., 2011). Second, numbers of adult thymus-derived CCR6^{+}CD27^{-} γδT17 cells were very low, sometimes "virtually absent" but never actually zero. Although tightly regulated, some individual CCR6^{+}CD27^{-} γδT17 cells were thus able to circumvent a potential mechanism restricting their development in adult thymi.

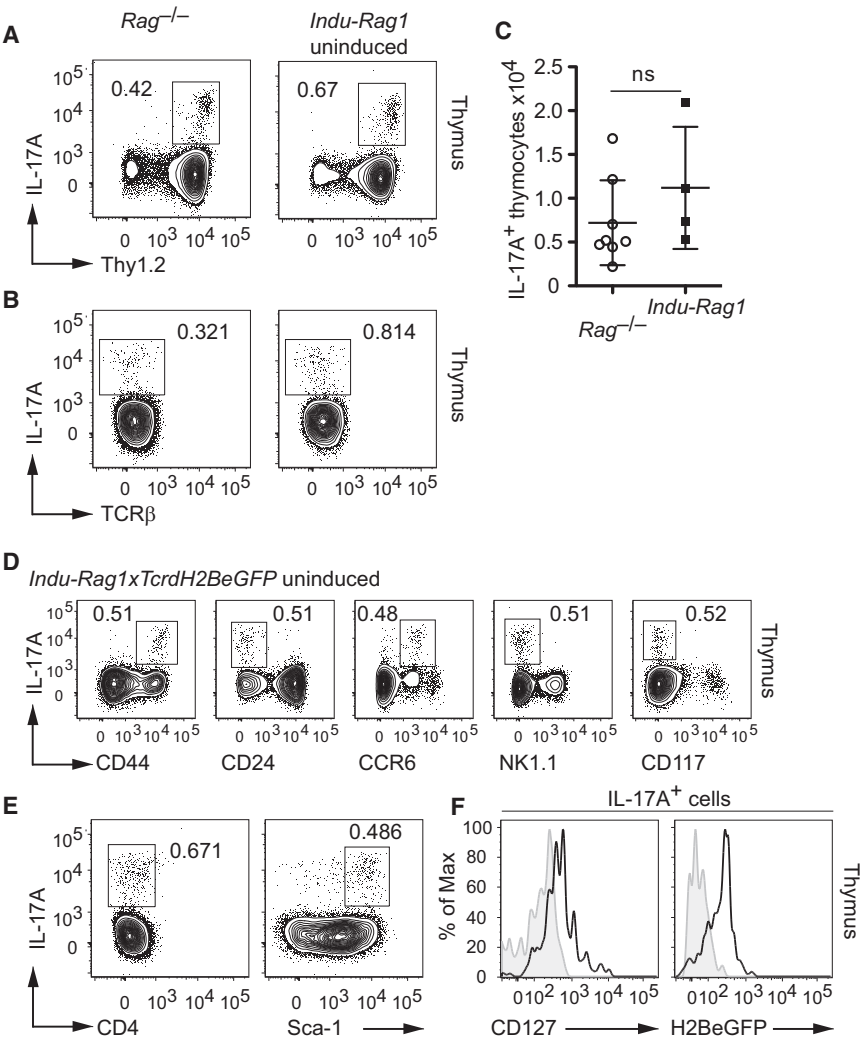

Figure 7. Innate Lymphoid Cells Produce IL-17 in the Thymus of Rag1-Deficient Mice

(A and B) Thymocytes from *cre*-negative Indu-*Rag1* × *TcrdH2BeGFP* mice (*Rag1*-KO) (A, B) and Indu-*Rag1* × *TcrdH2BeGFP* mice (Indu-Rag1 uninduced, Cre-positive) (A–F) were stained for the indicated surface markers and plotted versus intracellular IL-17A after PMA and ionomycin stimulation for 4 hr in the presence of brefeldin A.

(C) Scatter plot shows total numbers of IL-17A$^+$ thymocytes in *Rag*-KO compared to Indu-*Rag1* × *TcrdH2BeGFP* mice; each point is one mouse, mean ± SD and Mann-Whitney test is shown.

(D and E) Contour plots show intracellular staining of thymocytes for IL-17A versus indicated surface markers.

(F) Histograms show CD127 or *H2BeGFP*-reporter gene expression of IL-17A$^+$ thymocytes from uninduced Indu-*Rag1* × *TcrdH2BeGFP* mice. Grey overlays are "fluorescence minus one" (FMO) controls. Mice were 10–14 weeks old.

Data are representative of three (A, B, D–F) independent experiments with two to four mice per group.

It is conceivable that IL-17-producing αβ T cells negatively regulate γδT17 cell development because the latter partially occurred in mixed chimeras lacking IL-17-proficient αβ T cells. A potential negative feedback involving IL-17 is further supported by the data showing that the complete absence of IL-17 in *Il17af*$^{-/-}$ mice boosted the development of Rorγt$^+$ CCR6$^+$CD27$^-$ γδ T cells with a phenotype of "wannabe IL-17 producers."

Development of γδT17 cells was also not observed in adult Indu-*Rag1* × *TcrdH2BeGFP* mice. There, not only mature T cells but also double-positive CD4$^+$CD8$^+$ thymocytes were absent before *Rag1* induction. Hence, it is fair to state that αβ Th17 cells were not required to suppress γδT17 cell development in that model. Other sources of thymic IL-17 may have contributed to directly or indirectly inhibit γδT17 cell development. Indeed, we were able to identify a thymic population of IL-17-producing innate lymphoid cells. It remains to be determined how far these thymocytes overlap with a very recently described population of thymic population of IL-22-producing innate lymphoid cells (Dudakov et al., 2012). Also, it is not clear whether they might be genuine thymic precursors of Thy1$^+$ Sca-1$^+$ innate IL-17 producers found in the intestine of *Rag1*$^{-/-}$ mice (Buonocore et al., 2010). A resulting important question is to what extent such IL-17-producing innate lymphoid cells are present in Rag1-proficient animals. At least in part, analysis of fetal thymi from *TcrdH2BeGFP* mice gave a clue. There, already at day E15.5 a large fraction of thymic IL-17-producing cells showed intermediate H2BeGFP-reporter fluorescence, indicative of an open *Tcrd* locus ready for TCR-V(D)J rearrangements. However, we do not formally establish any precursor-product relationship between CD44^{hi}GFP$^-$ or CD44^{lo}GFP^{int} thymocytes and γδT17 cells. The former IL-17$^+$ populations may make abortive rearrangements and could constitute a dead end in T cell differentiation. Nevertheless, it is tempting to speculate that early embryonic thymocytes acquire the capacity to produce IL-17 prior to and independent of TCR rearrangement.

The idea that acquisition of IL-17 production capacity and γδTCR rearrangement are not linked is consistent with previous conclusions that γδT17 cell development is restricted to "antigen-naive" cells (Jensen et al., 2008). In fact, positive TCR selection rather commits thymic γδ T cells to an IFN-γ-producing effector fate characterized by suppression of Sox13 and Rorγt and induction of Egr3 (Turchinovich and Hayday, 2011). Accordingly, DETCs, which are a prototype of "ligand-experienced" γδ T cells (Jensen et al., 2008; Lewis et al., 2006; Xiong et al., 2004), explicitly lack the potential to produce IL-17 and rather secrete IFN-γ upon stimulation. Of note, quite the opposite seems to be true for selection of natural IL-17-producing αβ T cells, which are suggested to require a self-reactive TCR during thymic development (Marks et al., 2009).

When correlating a γδT17 cell effector fate with the absence of TCR signaling or selection, it is noteworthy that intestinal γδ intestinal epithelial lymphocytes (IELs) lack the potential to make IL-17 (Malinarich et al., 2010) although there is no evidence for positive selection of intestinal γδIELs (Jensen et al., 2009). This leaves three possibilities, namely either (1) intestinal γδIELs mature outside the thymus (Lambolez et al., 2006) where signals required for precommitment to IL-17 production are missing, (2) they undergo positive TCR selection, or (3) thymic γδIEL development occurs too late to provide embryonic imprinting toward IL-17-producing capacity. At present, it is unclear which TCR-independent signals are required to induce γδT17 cells in the embryonic thymus. Signals via TGF-β (Do et al., 2010), B cell-specific Src family kinase, B lymphoid kinase (Laird et al., 2010), RelA and RelB transcription factors (Powolny-Budnicka et al., 2011), and the Notch-Hes1 pathway (Shibata et al., 2011) have been shown to contribute. It remains to be determined whether γδT17 cell development and function depends on the same network of essential transcription factors regulating Th17 cell specification, i.e., Rorγt, STAT3, IRF-4, and BATF (M. Ciofani and D. Littman, personal communication).

In summary, our study demonstrates that only an embryonic thymus provides conditions that can prewire γδ T cells to IL-17 production. The underlying mechanisms are probably independent of TCR rearrangement and by extrapolation also of TCR selection. Although γδT17 cells are self-renewing and persistent, they may have evolved as a first set of innate IL-17-producing T cells that is progressively replaced by adaptive T cells during the first decade after birth. This hypothesis may reconcile the different roles ascribed to γδT17 cells in mice and young humans as compared to human adults.

EXPERIMENTAL PROCEDURES

Mice

8- to 14-week-old mice were used throughout the study; all mice were on C57BL/6 genetic background. Experiments were carried out according to institutional guidelines approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit. *Il17af*$^{-/-}$ mice were generated with C57BL/6 Bruce4 ES cells as illustrated in Figure S1. In contrast to a recent report on a related model (Ishigame et al., 2009), *Il17af*$^{-/-}$ mice appeared healthy and showed no opportunistic infections. This discrepancy may be explained by different housing conditions or different genetic backgrounds. *TcrdH2BeGFP* mice have been described (Prinz et al., 2006). Indu-*Rag1* mice (Düber et al., 2009) were crossed to *TcrdH2BeGFP* to obtain tamoxifen-inducible Indu-*Rag1* × *TcrdH2BeGFP* mice. Conditional *Rag1* expression was facilitated by introducing one allele of *Rosa26creERT2* (Hameyer et al., 2007).

Generation of Bone Marrow Chimeras and Fetal Liver Chimeras

Recipients for the generation of chimeras were lethally (9 Gy) irradiated *Il17af*$^{-/-}$ mice. 1–2 × 10^7 lin$^-$ bone marrow cells were injected into the lateral tail vein within 24 hr postirradiation. Bone marrow cells were isolated from femurs and tibia of *C57BL/6* (Thy1.2), *B6.129S2-Tcra* < *tm1Mom* > */J* (Thy1.2), C57BL/6-*TcrdH2BeGFP* (Thy1.2), and C57BL/6 *IL17af*$^{-/-}$ (Thy1.1 or Thy1.2) mice. Fetal liver from embryonic day 13.5 was isolated from *TcrdH2BeGFP* mice and single-cell suspensions (300 μl per fetal liver) prepared.

Embryonic Thymus Transplantation

Fetal embryonic day E15–E16 thymic lobes from CD45.1$^+$ WT mice were transplanted under the kidney capsules of Indu-*Rag1* × *TcrdH2BeGFP* mice (CD45.2$^+$). *cre* expression in recipient mice was induced by tamoxifen gavage 1 hr before transplantation. Animals were treated with prophylactic antibiotics (Cotrimoxazol) and pain killers (Novaminsulfon; ratiopharm) during the initial 2 weeks.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.06.003.

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